Carnitine/xenobiotics transporters in the human mammary gland epithelia, MCF12A

Bruce Kwok,1,2 Ariko Yamauchi,1,2 Ratheishan Rajesjan,1,2 Lillian Chan,1,2 Upinder Dhillon,1,2 Wen Gao,1,2 Haibo Xu,1,2 Bernice Wang,1,2 Shinichiro Takahashi,1,2 John Semple,2,3 Ikumi Tamai,4 Jun-Ichi Nezu,5 Akira Tsuji,4 Patricia Harper,1,2 and Shinya Ito1,2

1Division of Clinical Pharmacology and Toxicology, Department of Pediatrics, Research Institute, Hospital for Sick Children, 2University of Toronto, Toronto, Ontario, Canada; 3Department of Plastic Surgery, Sunnybrook and Women’s College Health Sciences Centre, Toronto, Ontario, Canada; 4Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, and 5Chugai Pharmaceutical Company Limited, Ibaragi, Japan

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MANY MOTHERS CHOOSE breastfeeding, which is the best feeding method for neonates. However, the majority of these women receive medications in the postpartum period (22). As a result, newborns have increasing likelihood of being breastfed by mothers on medications.

The mammary gland is regarded not only as a milk-processing organ but also as a de facto protective barrier for the infant, enhancing accumulation of nutrients and endogenous bioactive factors in milk, while limiting transfer of nonnutritional, potentially toxic substances. Indeed, in contrast to excreta such as urine, xenobiotic/toxicant concentrations in milk are usually lower than those of maternal serum (16). However, this barrier function breaks down if challenged with certain compounds of cationic nature, which causes their accumulation in milk.

Cationic drugs constitute an important group of drugs that tend to be concentrated in milk in relation to maternal plasma levels, causing sometimes toxicity in breastfed infants (21 and 31; reviewed in Ref. 18). Accumulation of cationic drugs in milk was believed to be mainly the result of ion trapping as a result of relative acidity of milk compared with maternal plasma. A concept behind this is the long-held view that drugs are excreted in milk almost exclusively by passive diffusion. However, McNamara et al. (23, 24) showed that excretion of organic cations such as cimetidine and ranitidine (24) in milk of rats is more than expected from simple diffusion and ion trapping. An in vivo human study by the same group has also shown that accumulation of cimetidine in milk is significantly higher than expected from passive diffusion (29). However, the molecular mechanisms are not fully understood.

Recently, the breast cancer resistance protein BCRP (ABCG2), which belongs to the ATP-binding cassette transporter family, has been identified as a mammary drug/toxin transporter (19). Namely, the Abcg2−/− mice showed significantly reduced milk excretion of certain drugs and toxins such as nitrofurantoin, cimetidine, acyclovir, and topotecan (19, 25). However, roles of other drug-transporting proteins for a wide range of cationic compounds remain to be elucidated in the human mammary gland.

Organic cation transporters (OCTs) are essential in eliminating nonnutrient, often toxic, cationic compounds that are ingested as food or drug. These proteins are classified as the solute carrier superfamily (SLC). Grundemann et al. (12) cloned the first member of the OCT family from the rat kidney. In humans, two homologous transporters, human (h) OCT1 (SLC22A1) and OCT2 (SLC22A2), were the first OCTs cloned...
OCT2 is a unique transporter with a dual mode of transport (one as a Na\(^{+}\)-independent OCT and the other as a Na\(^{+}\)-dependent, high-affinity carnitine transporter). Human milk is rich in carnitine (30), which is essential in the β-oxidation of long-chain fatty acids. Neonates are developmentally immature for carnitine biosynthesis, depending on breast milk as an exogenous source of carnitine. Moreover, a congenital defect of hOCTN2 causes a lethal metabolic condition known as primary systemic carnitine deficiency (27). This implies an important role of carnitine transporters in the human mammary gland.

In accordance with the in vivo data suggesting carrier-mediated excretion of cationic drugs in milk, mammary gland mRNA expressions of the SLC OCTs have been reported recently in rats (9) and humans (1). However, the function of these gene products in the human mammary gland remains to be demonstrated. Moreover, it is intriguing that the mammary gland expresses xenobiotic transporters of detoxification function, including BCRP, because the notion apparently contradicts the nutritional role that the mammary gland plays for the offspring. It is possible that nutrient carriers such as OCTN2 are hijacked by potentially toxic cationic xenobiotics, causing collapse of the barrier function of the mammary gland epithelium.

To gain insight into an integrated mechanism of drug excretion in the human mammary gland, our objective in the present study was to demonstrate and characterize carrier-mediated transport of organic cations in the MCF12A human mammary gland epithelial cells, which are widely used as an in vitro system of noncancerous human mammary gland epithelia. In particular, we asked questions whether OCTN2 as a representative SLC transporter of dual roles [i.e., transport of a nutrient (l-carnitine) and cationic xenobiotics] is expressed and functioning in the human mammary gland and, if so, whether therapeutic compounds interfere with its function. Our data indicate mammalian expression of multiple SLC OCTs, including OCTN2, and corresponding functional multiplicity for uptake of carnitine and tetraethylammonium (TEA). Overall, carnitine transport in the MCF12A cells is a robust process, requiring superatherapeutically concentrations of cationic drugs for its inhibition. In addition to the high-affinity carnitine uptake compatible with OCTN2 function, partial Cl\(^{-}\) dependency of the uptake suggests a functional role of a Na\(^{+}\)/Cl\(^{-}\}-dependent low-affinity carnitine transporter, human amino acid transporter B\(^{0+}\) (ATB\(^{0+}\); see Refs. 26 and 33). In agreement with the notion, the MCF12A cells show specific uptake of glycine, an ATB\(^{0+}\) substrate, which is inhibited by carnitine and cationic drugs such as verapamil and desipramine.

**Materials and Methods**

**Cell culture.** The MCF12A human mammary gland epithelial cell line was obtained from American Type Culture Collection (Rockville, MD) at a passage 53. They were cultured, as specified by ATCC, in 1:1 mixture of Ham’s F-12 medium and DMEM with 5% horse serum, supplemented with 2 mM glutamine, 0.1 μg/ml choloroenteroxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 20 ng/ml epidermal growth factor. The cells were maintained under an atmosphere of 95% air-5% CO\(_2\) at 37°C and subcultured weekly using 0.20% EDTA and 0.05% trypsin. Media were changed every 2-3 days. For the uptake experiments, the MCF12A cells were used in the passages 50-60.

A human mammary myoepithelial cell line (HMEC) was obtained at the passage 7 from Clonetics (San Diego, CA). The cells were maintained in the Mammary Epithelial Cell Basal Medium (Clonetics) supplemented with 52 μg/ml bovine pituitary extract, 10 ng/ml human recombinant epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B. The cell line was maintained under an atmosphere of 95% air-5% CO\(_2\) at 37°C and subcultured two times weekly using 0.02% EDTA and 0.05% trypsin. The HMEC cells were used at the passage 10.

**Human tissue preparation.** Discarded mammary gland tissue was obtained from women of child-bearing age undergoing cosmetic breast reduction operation after the consent form was obtained. The study protocol was approved by the respective Institutional Research Boards. We processed the tissue as previously described (38). Briefly, the tissue was dissected, immediately after surgery; excess adipose tissue was removed, and the parenchyma containing the ductal-lobular-alveolar structures was isolated and processed for total RNA extraction. The remaining tissue was snap-frozen in liquid nitrogen and stored at −80°C until used for protein detection. Mammary tissue used for immunohistochemistry was fixed with 10% formalin in PBS immediately after surgery, subsequently embedded in paraffin, and sectioned to ~5 μm thickness. Human kidney tissue samples were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute.

**RT-PCR.** Total RNA was extracted from cultured cells (e.g., MCF12A cells and HMEC cells), dissected glandular tissue of the mammary gland, and other tissue samples, using the TRIzol reagent (GIBCO-BRL, Grand Island, NY), and cDNA was synthesized by reverse transcription using Moloney murine leukemia virus RT. For RNA extraction from the mammary tissue, homogenized tissue was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was then processed for total RNA extraction as described above. Purity and integrity of the RNA samples were verified using an ultraviolet spectrophotometer and agarose gel visualization of ribosomal bands, respectively. The respective transcripts were then amplified using the primers. For OCT1 and OCT2, we used the primers reported by Gorboulev et al. (11). We designed specific primers for hOCTN1 (forward: 5'-CTG GAT CCT CCT AAT TTA CAT GG-3'; reverse: 5'-AGG AGA CTC TCT AAG AAT GAT TGG-3'), hOCTN2 (forward: 5'-GTG CTG TTG GGC TTC TCC ATT TA-3'; reverse: 5'-AGC TGC ATG AAG AGA AGG ACA CTG-3'), EMT/hOCT3 (forward: 5'-ACT CCA CCA TCG TCA GCG AG-3'; reverse: 5'-CTG GAT TGG CCA GGA GTT GG-3'), and ATB\(^{0+}\) (forward: 5'-ACA CAT GGC CCA CAT ATC TGG-3'; reverse: 5'-ATG GAA TTG CGC CAT AAT TAG G-3'). The respective amplification products are as follows: hOCTN1, 785 bp, corresponding to the nucleotide positions 1197-1738; hOCTN2, 800 bp, covering the nucleotide positions 580-1379; EMT/hOCT3, 404 bp, covering the nucleotide positions between 437 and 840; and ATB\(^{0+}\), 541 bp, corresponding to the positions 1197-1738. In addition, we designed a specific primer for Fly-like putative transporter (Flip) 2/carnitine transporter (CT) 2 (forward: 5'-GGA GTC ACC CCT CAT CAT GT-3'; reverse: 5'-CAA AAG TCA CCG ATC CCA GT-3'). It can distinguish CT2 and its splice variant,
Flipt2/OCT6, because the primer covers the Flipt2/OCT6-specific sequence, which is an in-frame retention of the third intron of CT2 gene (6, 7, 10). The expected amplification products for Flipt2/OCT6 and CT2 are 385 and 289 bp, respectively. Because the primer does not encompass an intronic sequence of Flipt2/OCT6, we ruled out genomic contamination by performing PCR without using reverse transcriptase.

The identity of each PCR product was verified by sequencing (Center for Applied Genomics, the Hospital for Sick Children, Ontario, Canada).

**Immunoblot analyses for human OCTN1 and OCTN2.** Protein was extracted from the mammary tissue, the kidney, and the cell-membrane fraction of the MCF12A cells. Briefly, the mammary gland and the kidney tissues were homogenized in ice-cold RIPA buffer containing phenylmethylsulfonyl fluoride and protease inhibitors. After incubation for 1 h on ice, the lysate was centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was collected. For MCF12A cells, cell membrane fractions were made as previously described (2), with some modifications. Briefly, MCF12A cells (50 × 10⁶) were suspended in 10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 200 μg/ml EDTA with protease inhibitors, 25 μg/ml RNase, and 50 μg/ml DNase I. After 10 min of incubation, cells were homogenized using a glass homogenizer with 100 strokes. The homogenate was centrifuged at 800 g for 15 min at 4°C. The supernatant was centrifuged at 100,000 g for 20 min at 4°C. The pellet containing the enriched membrane fraction was then resuspended in 10 mM Tris-HCl, pH 7.4, with 125 mM sucrose and protease inhibitors. Protein concentration was measured by the BCA Protein Assay kit (Pierce, Rockford, IL) using BSA as a standard.

We used specific, site-directed polyclonal antibodies raised against the COOH termini of human OCTN1 and OCTN2. The amino acid sequences of the antigenic peptides were WFRSGKKTRDSME-TEENPK (corresponding to amino acid residues 527–545) for hOCTN1 and HRKTPSHTRMLKDGGQERP (corresponding to amino acid residues 532–550) for hOCTN2 (37).

Protein samples (50–100 μg) were loaded on a 7.5% SDS-polyacrylamide gel, subjected to electrophoresis, and transferred to the nitrocellulose membranes that were then blocked with 4% skim milk overnight at 4°C. The membranes were incubated with the primary antibodies (1:1,000 dilution) for 3 h at room temperature. After thorough rinsing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (CSA rabbit HRP-7000) and rehydrated human breast tissue sections were treated to inactivate endogenous peroxidase and biotin. Slides were washed and incubated with the preadsorbed antibodies at 4°C overnight. The preadsorbed antibodies corresponding antigenic peptides were incubated in a 5:1 ratio with the primary antibodies at 4°C overnight. The preadsorbed antibodies were then used with the protein-loaded membranes.

**Immunohistochemical detection of human OCTN2.** Deparaffinized and rehydrated human breast tissue sections were treated to inactivate endogenous peroxidase and biotin. Slides were washed and incubated for 1 h at room temperature with the rabbit polyclonal antiserum (37) at 1:80 dilution in Tris-buffered saline containing 0.1% Tween 20 (TBST). After being washed in TBST, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (CSA rabbit link; Dako) for 45 min, washed in TBST, and developed for 1–3 min in diaminobenzidine solution and hydrogen peroxide. After the color was developed, the slides were counterstained with Harris Hematoxylin stain. Preimmune rabbit serum was used as controls.

**Uptake experiments.** MCF12A cells in the passages between 56 and 60 were grown on six-well tissue culture plates at an initial seeding density of 5 × 10⁴ cells/well until 80–90% confluency in the same culture condition as described above. After the media was removed, the cells were immersed in uptake media. For uptake experiments, the media (pH 7.5) consisted of 25 mM Tris/HEPES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, and 5 mM glucose (8). After preincubation at 37°C in room air for 60 min, 65 μM [14C]TEA with 25 nM [3H]mannitol or 0.1 μM [3H]carnitine with 0.86 μM [14C]mannitol (to allow dual counting at the probe-mannitol disintegrations/min ratio of 10:1) were added to the uptake media in the presence (nonspecific uptake) and absence (total uptake) of an excess amount of the unlabeled substrates at various concentrations of inhibitors. One hundred-fold excess of unlabeled carnitine or TEA (up to 10 mM because of solubility) was used to determine the nonspecific uptake for the labeled substrates. The nonspecific uptake values were subtracted from the total uptake values to obtain the specific uptake. At a given incubation time, the incubation medium was removed, and the cells were rinsed two times in ice-cold PBS for 30 s each. The cell membranes were then solubilized using 0.1% Triton X-100 (BDH, Toronto, Ontario), and the radioactivity was counted as disintegration/min. The cellular protein content was measured using the BCA protein assay kit (Pierce, Rockford, IL) and BSA as the standard. For Na⁺-, free experiments, NaCl was substituted with equimolar LiCl, KCl, or choline chloride. Similarly, for Cl⁻, free experiments, Cl salts were replaced with equimolar sodium gluconate, sodium thiocyanate, or sodium phosphate. For pH-dependency experiments, the pH was adjusted appropriately using HCl or NaOH (36). [3H]glycine (1 μM) uptake was examined similarly in the presence and absence of various inhibitors using the uptake buffer reported previously (26). All experiments were done in triplicate.

**Data analyses.** Data were compared using Student t-test or ANOVA (followed by Tukey’s test), where appropriate. In the text, results were expressed as means ± SE of 3–9 independent measurements. In Figs. 1–7, error bars were specifically designated whether they represent SD or SE.

**Estimation of kinetic parameters.** Kinetic parameters of carnitine and TEA uptake were determined by fitting the pooled data on the...
total and nonspecific initial uptake rates at various substrate concentrations to the Michaelis-Menten models. We tested three different models with one, two, or three Michaelis-Menten terms, all of which had a linear term to take into account a nonspecific diffusion/uptake component. The two-system model as a representative example is shown below:

\[
\text{total uptake} = [C] \times \left( \frac{V_{\text{max}1}([C]_1 + [C])}{K_{\text{m}1} + [C]} + \frac{V_{\text{max}2}([C]_2 + [C])}{K_{\text{m}2} + [C]} + A \right)
\]

where suffixes 1 and 2 denote system 1 and 2, respectively, \([C]\) is the substrate concentration, and \(A\) represents the nonspecific uptake constant. The experimentally determined nonspecific uptake was also fit to the following equation simultaneously, so that the parameters satisfying both equations at the same time can be estimated:

\[
\text{nonspecific uptake} = [C] \times A
\]

The nonlinear data fitting was performed using MLAB (Civilized Software, Bethesda, MD), as described elsewhere (17). Briefly, after confirming that there is no systematic deviation in data fitting, and that errors for individual parameter estimates are relatively small, the statistical criteria were used for the selection of a best-fit model. The residual sum-of-square error (RSS) was compared between models using the \(F\)-test. If there is a statistically significant difference (i.e., \(P < 0.05\)), the model with a greater sum of square error was rejected in favor of the other. A best-fit model requires the fewest parameters, while providing a reasonably good fitting that is statistically indistinguishable from that of more complex models. This aspect was assessed by two parsimony criteria; namely, the Akaike’s information criterion (AIC) and the Schwartz criterion (SC). In the case where RSSs of two models are not statistically different, a model with lower AIC and SC was selected as a better model.

Materials. Insulin, epidermal growth factor, choler toxin, and amino acids were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Quinidine was a gift from AstraZeneca. Cell culture-related materials were obtained from GIBCO-BRL. \(^{1-}[N\text{-methyl-}^{3}\text{H}]\)carnitine hydrochloride (80 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). \(^{1-}[1\text{-}{}^{13}\text{C}]\)mannitol (58.1 mCi/mmol), \(^{1-}[1\text{-}{}^{13}\text{H}(N)]\)mannitol (26.3 Ci/mmol) were purchased from New England Nuclear Life Science Products (Boston, MA). \(^{2-}[3\text{H}]\)glycine (60 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA).

RESULTS

mRNA expression of OCTs. We examined the expression of OCTs in the three representative models of human mammary gland: the mammary gland epithelial cell line (MCF12A), the mammary cell line with myoepithelial cell characteristics (HMEC), and the human mammary gland tissues obtained from women undergoing breast reduction surgery. In all three, we detected PCR products for hOCT1, hOCTN2, hOCTN1, and hOCT3/EMT (Fig. 1A). ATB\(^{0+}\), a Na\(^{+}/\text{Cl}^{-}\)-dependent
amino acid transporter, was also detectable in MCF12A, the breast tissue, and in kidney (Fig. 1B). Sequence analysis further confirmed the identity (data not shown). However, RT-PCR for hOCT2 produced no amplicon (Fig. 1A).

Another high-affinity carnitine transporter (CT2) has been recently reported as the testis-specific protein by Enomoto et al. (6). Interestingly, CT2 mRNA was undetectable in the MCF12A cells and the mammary gland tissues, but its splice variant showed ample expression (Fig. 1C), which was independently reported and designated as Fl ipt2 by Eraly and Nigam (7) because of its homology to Drosophila putative transporter (Fly-like putative transporter 2), and OCT6 by Gong et al. (10). PCR without RT was negative, indicating no genomic DNA contamination (data not shown).

**Immunoblotting.** The affinity-purified OCTN1 and -2 antibodies against the corresponding peptides stained bands with a relative molecular mass of ~60 kDa (Fig. 2, A-C). The estimated sizes of OCTN1 and -2 are ~63 kDa, but the detected bands were consistently smaller than 63 kDa, suggesting post-transcriptional modification and/or degradation. Noteworthy is that a recent study on hOCTN2 expression in Caco-2 intestinal cells reported similar results (5).

**Immunohistochemistry with hOCTN2 antibody.** To determine mammary tissue localization of hOCTN2, normal human mammary tissues were stained with the specific hOCTN2 antibody (36, 37). In accordance with the data obtained by RT-PCR and Western blotting, staining was evident in the ductular-lobular-alveolar structures in the mammary gland parenchyma, especially in the alveolar glands (Fig. 2D). Specificity of immunostaining was established by observing a lack of staining with the preimmune rabbit serum in the same tissue. These results indicate that OCTN2 expression is mainly localized in the alveolar epithelia of the mammary gland. Immunohistochemistry using the hOCTN1 antibody was unsuccessful.

**L-carnitine and TEA uptake by MCF12A.** The time course of L-carnitine uptake (Fig. 3A) indicated a linear phase for the initial 120 min. TEA uptake in MCF12A cells was time dependent, with the initial linear phase of ~60 min (Fig. 3B). On the basis of these results, subsequent experiments for initial uptake rate of L-carnitine and TEA were conducted at 60 and 30 min, respectively.

Fig. 3. Time course of L-carnitine and tetraethylammonium (TEA) uptake by MCF12A human mammary gland epithelia. L-[14C]carnitine (0.1 μM) or [14C]TEA (65 μM) was added to the incubation media, and uptake by MCF12A cells was measured over various time points. Uptake was nearly linear for 120 min for carnitine (A) and 60 min for TEA (B). The results are shown as means and SE of 3 different experiments.

![Graph A](image1.png)

**Fig. 4.** Determinants of carnitine and TEA uptake. A: Na+/Cl− dependency of carnitine uptake. L-[H3]carnitine (0.1 μM) uptake was measured in MCF12A for 60 min. When Na+ in the medium was replaced with other monovalent cations, the uptake was inhibited significantly. When Cl− was replaced with other anions, carnitine uptake was moderately reduced, indicating that the carnitine uptake function of the MCF12A cells is at least partly dependent on Na+ and Cl−. The results are expressed as %control uptake in the presence of sodium chloride, representing the mean and the SE of 3–4 different measurements. Ch, choline; CN, thiocyanate; Glu, gluconate; Ph, phosphonate. *P < 0.05 compared with control (see RESULTS). B: pH dependency of TEA uptake. [14C]TEA (65 μM) uptake in MCF12A cells was measured at varying pH for 1 h. As pH increased from 6 to 8, TEA uptake was increased by ~60%. Results are expressed as %TEA uptake at pH 6, depicting the mean and SE of 3 experiments. *P = 0.011 (see RESULTS).
l-Carnitine uptake in MCF12A cells was Na⁺ dependent. When Na⁺ in the medium was replaced with other monovalent cations, the uptake was inhibited significantly [ANOVA: F = 7.75; degrees of freedom (df) = 6, P < 0.001; Fig. 4A]. Choline exerted the highest inhibition (P < 0.001), followed by potassium (P = 0.006) and lithium (P = 0.018). When Cl⁻ was replaced (Fig. 4A), ~40% inhibition was observed. However, only sodium phosphate reached statistical significance (P = 0.035), which is likely to be because of lack of power of the sample size. Overall, the results suggest that the carnitine uptake function of the MCF12A cells is at least in part dependent on Na⁺ and Cl⁻. TEA uptake was pH dependent (Fig. 4B), increasing by ~60% as media pH was increased from 6 to 8 (ANOVA: F = 9.96; df = 2, P = 0.012).

Kinetics of organic cation uptake in MCF12A. To gain insight into the functional roles of these transporters, different Michaelis-Menten models were fit to the saturation data for carnitine and TEA. Because a model with more than three Michaelis-Menten systems did not converge to meaningful estimates, we examined the following two simpler models with a nonspecific diffusion term: a single-system model and a double-system model. None of the fitting results of the two models for either substrate was under active constraints at termination. Although the modeling analyses were successfully employed, it should be noted that experimental constraints did not allow us to increase the substrate concentrations to cover the Kₘ values of the low-affinity systems.

For l-carnitine (Fig. 5A), the single-system model caused systematic deviation from the data points (the runs test; data not shown) and was rejected in favor of the double-system model (r² = 0.999; RSS = 5.96). The double-system model predicted presence of a high- and a low-affinity system with Kₘ of 5.1 ± 4.0 μM (Vₘₐₓ = 832 ± 246 pmol·mg protein⁻¹·h⁻¹) and 15.9 ± 6.9 mM (Vₘₐₓ = 170 ± 59 nmol·mg protein⁻¹·h⁻¹), respectively. Inhibition of 0.1 μM l-[³H]carnitine uptake in MCF12A cells was demonstrated using various cationic compounds (Fig. 5B). Verapamil and l-carnitine itself at a 20–50 μM range significantly inhibited l-carnitine uptake. Other therapeutic drugs such as cimetidine, carbamazepine, quinidine, and desipramine showed >50% inhibition at supratherapeutic concentrations. l-Carnitine transport by ATB0⁺, a Na⁺/Cl⁻-dependent transporter for neutral and cationic amino acids, is known to be inhibited by zwitterionic and cationic amino acids such as alanine, glycine,
For TEA (Fig. 6A), the difference in goodness-of-fit of the two models did not reach statistical significance ($F = 6.44$, $P = 0.056$), although the double-system model resulted in substantially lower RSS (0.82 vs. 3.48). Given the statistically indistinguishable fitting performance, the parsimony criteria (AIC and SC) were used to select the best model. AIC and SC of the double-system model (AIC, 8.3; and SC, 9.2) were nearly one-half those of the single-system model (AIC, 17.2; and SC, 17.8); the AIC and SC differences between the two models were about eight, indicating the strong support for the former. Therefore, we considered the double-system model to also be the best model for TEA. This model predicted that the high-affinity system has $K_m$ of 1.63 ± 0.51 mM and $V_{max}$ of 8.9 ± 2.4 nmol·mg$^{-1}$·h$^{-1}$, and that a low-affinity system has $K_m$ of 73.6 ± 21.6 mM and $V_{max}$ of 139.5 ± 20.0 nmol·mg$^{-1}$·h$^{-1}$. The uptake of 65 μM $[^{14}C]$TEA was inhibited by various drugs (Fig. 6B). Of the therapeutic drugs tested, desipramine and disopyramide were the most potent, inhibiting >50–80% of the uptake at concentrations of 10–100 μM, followed by cimetidine. In contrast to carnitine uptake inhibition, TEA uptake was not inhibited by carnitine at 1–5 mM.

Glycine uptake in MCF12A. If both hOCT2 and ATB0$^+$ contribute to the mammary carnitine uptake as our results suggest, it is intriguing that drugs such as verapamil and desipramine exert nearly complete inhibition of carnitine uptake (Fig 5B). This implies that these drugs may interfere not only with hOCT2 but also with ATB0$^+$.

Therefore, we have examined inhibitor specificity of ATB0$^+$ in the MCF12A cells, using glycine as a probe substrate (15, 26). As shown in Fig. 7, 1 μM $[^{3}H]$glycine uptake was significantly reduced by all inhibitors but aspartic and glutamic acid (ANOVA: df = 13, $F = 47.2, P < 0.001$; pairwise comparison with no inhibitor without 1 mM unlabeled glycine by Tukey’s test, $P < 0.001$). Unlabeled glycine (1 mM) decreased the uptake of 1 μM $[^{3}H]$glycine by ~60%, indicating presence of the specific glycine uptake component in the MCF12A cells, supposedly through ATB0$^+$.

In the presence of 1 mM unlabeled glycine, however, carnitine and verapamil did not show significant additional inhibition on 1 μM $[^{3}H]$glycine uptake, indicating that they inhibit the glycine-specific component (i.e., through ATB0$^+$). Asparagine and glutamine also decreased the 1 μM $[^{3}H]$glycine uptake ($P < 0.001$), but, in the presence of 1 mM unlabeled glycine, they further decreased the 1 μM $[^{3}H]$glycine uptake significantly ($P = 0.003$ and 0.015, respectively), probably because of their relatively low affinity to human ATB0$^+$ (33) and/or interaction with a non-ATB0$^+$ low-affinity glycine transporter. Consistent with the data on mouse and human ATB0$^+$ expression systems (26, 33), aspartate and glutamate showed virtually no inhibition. Interestingly, 1 mM desipramine showed substantial inhibition, even compared with 1 mM unlabeled glycine.

**DISCUSSION**

Our study showed expression of several SLC OCTs, and corresponding multiplicity of carnitine/TEA uptake kinetics in the MCF12A human mammary gland epithelial cells. Although vectoral transport of drugs across a polarized cell layer of the mammary gland epithelia remains to be examined, our data suggest that excretion of cationic drugs in human milk is most likely mediated by a network of multiple drug-transporting proteins.

It should be noted that our model-derived $K_m$ values are probably hybrid constants and that further experimental validation of the low-affinity systems was not possible. In addition, phosphorylation status of the transporters such as OCT1 infl-
Fig. 7. Inhibition of 1 mM [3H]glycine uptake. [3H]Glycine (1 mM) uptake was measured in MCF12A cells for 60 min in the presence and absence (control: open bars “no inhibitor”) of various inhibitors at 1 mM. Additional effects of 1 mM unlabeled glycine were also examined similarly in the presence and absence of other inhibitors. All inhibitors but aspartate and glutamate (stippled bars) significantly decreased the 1 mM [3H]glycine uptake (P < 0.001). Addition of 1 mM unlabeled glycine to carnitine, verapamil, and desipramine (hatched bars) did not significantly alter the inhibition. Asparagine and glutamine (solid bars) further decreased the uptake in the presence of 1 mM unlabeled glycine. Results are expressed as % control (no inhibitor without unlabeled glycine) and are shown with the mean values and SE of 3 separate experiments.

CARNITINE/DRUG TRANSPORT IN THE HUMAN MAMMARY GLAND

Nakanishi et al. (26) recently reported the Na+/H+ exchange to be species specific: Na+/H+ gradient-dependent carnitine transport in rat mammary gland tissues at nonlactating stage. Presently, it is not known if its expression and function are decreased similarly in the plasma membrane of the intact mammary gland epithelia at the lactating stage. It is critical to examine protein expression of various transporters, including OCTN1, or OCTN2. Interestingly, the EMT/OCT3-mediated transport of TEA in those studies provides similar m values to our estimate (mouse: Km, 1.9 mM; rat: Km, 2.5 mM; and human: Ks, 1.3 mM; see Refs. 20 and 42).

Alcorn et al. (1) reported that hOCTN2 mRNA expression in the epithelial cells dislodged from the mammary gland in milk is substantially lower than that seen in the mammary gland tissues at nonlactating stage. Presently, it is not known if its expression and function are decreased similarly in the plasma membrane of the intact mammary gland epithelia at the lactating stage. It is critical to examine protein expression of various transporters, including OCTN2 in the in vivo lactating human mammary gland epithelia.

The inhibitor specificity assay in this study showed that several therapeutic drugs with cationic nature interact with the carnitine/TEA transporters in the mammary gland. However, their inhibitory constants, especially for the carnitine transport, appear relatively high compared with their therapeutic plasma concentrations, suggesting that the likelihood of in vivo drug-carnitine interaction is remote. Overall, carnitine transport in the mammary gland seems to be a robust process, which is not readily affected by exogenous xenobiotics.

Interestingly, Flp2/OCT6 (7, 10), a splice variant of CT2 (6), was detectable in the human mammary gland. CT2 is a high-affinity Na+/H+ exchange carnitine transporter with an estimated uptake Km of ~20 μM in a Xenopus oocyte expression system (6). A carnitine transport parameter has not been reported for Flp2/OCT6. Further studies are needed to reveal its exact functional role.
gesting their contribution to drug excretion in human milk. The MCF12A cells have a potential to be an in vitro mammary model for organic cation transfer in human milk, which circumvents ethically challenging drug studies in breastfeeding mother-infant dyads. To this end, it would be important to determine if the MCF12A cells undergo lactogenesis and highly express other transporters such as BCRP, which is known to be significantly upregulated during lactation.

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